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Eukaryotic Microbial Communities Associated with the Rhizosphere of the Temperate Fern *Thelypteris noveboracensis* (L.) Nieuwl.

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Abstract.—Microbial communities, associated with terrestrial mosses (Bryopsida) and the rhizosphere of agricultural and natural occurring seed plants, have been rather extensively examined; but less is known about associations with seedless vascular plants, including ferns. The New York fern (*Thelypteris noveboracensis*), typically found within deciduous forests, occurs in locally extensive stands in North America extending from northeastern Canada to southeastern U.S.A. Soil samples were obtained in autumn (2007) and early summer (2008) within a plot of *T. noveboracensis* in the understory of deciduous trees in the forest reserve at Torrey Cliff, NY to document the rhizosphere (root-associated) density of commonly occurring heterotrophic eukaryotic microbes (protozoa), including microflagellates, naked amoebae and testate amoebae. The ranges in densities (number/g soil dry weight) are as follows: microflagellates (6.5×10^6–1.3×10^8), naked amoebae (1.8×10^3–4.0×10^6) and testate amoebae (ca. 400). Very few active ciliates were observed. This is the first report of microbial communities associated with the rhizosphere of ferns and provides a step toward a more complete documentation of protozoa associated with plant communities. Some comparative data of protozoa associated with mosses and seed plants are also presented.

Key Words.—microbial diversity, microflagellates, naked amoebae, New York fern, soil biology, terrestrial ecology, testate amoebae

In recent decades, considerable ecological research has focused on understanding the coupling of aboveground and belowground processes; that is, how primary production of plant organic matter, including exudates from roots, affects the belowground soil microbial communities. Bacteria supported in part by organic matter from plants, and stimulated by the presence of eukaryotic microbes (protozoa) and secondarily by bactivorous nematodes, mineralize and recycle soil nutrients, thus enhancing plant growth. Therefore, understanding the functioning of land plants must include knowledge of the protozoan community in soils and the rhizospheres surrounding roots. Soil microbial communities associated with terrestrial mosses (e.g., Anderson, 2006, 2008) and the rhizosphere of agricultural and naturally occurring seed plants, have been extensively studied in a wide range of terrestrial locations (Bamforth, 1984; Clarholm, 1985, 1989; Foissner, 1987; Griffiths 1990; Cowling, 1994; Anderson, 2000; Adl, 2003; Li *et al.*, 2005). However, less is known about microbial communities associated with the rhizosphere of seedless vascular plants, including ferns; although, they are widely distributed geographically from arctic to tropical habitats (e.g., Moran, 2004). Fern rhizomes and roots, in addition to decaying shed fronds, are substantial

sources of organic matter that may support rich microbial communities. Fern root systems also may secrete possible allelopathic and antimicrobial substances (e.g., Horsley, 1977; Stetsenko *et al.*, 1984; Hill and Silander, 2001). Hence, a better understanding of microbial communities associated with fern rhizospheres is warranted to more fully document the microbial communities associated with a wide range of plant groups, and eventually to more completely understand the dynamics of the interactions of ferns with associated terrestrial eukaryotic microbes. The purpose of this research was to contribute to our knowledge of fern rhizosphere microbial communities by examining the eukaryotic microbes associated with the rhizosphere of *Thelypteris noveboracensis* (L.) Nieuwl., a widely distributed, temperate fern in eastern Canada and U.S.A.

Materials and Methods

Sample site.—Soil samples, using a LaMotte model EP corer, were obtained from the rhizosphere of a well-established plot of *T. noveboracensis* in the Torrey Cliff Forest Reserve at Palisades, NY (40°59′ 58″ N; 73° 54′ 30″ W). Three separate soil cores approximately 2 cm long were taken on each sampling date and combined to obtain a more representative soil sample. The mixed sample was placed in a sealed plastic bag and immediately returned to the Lamont-Doherty Observatory laboratory for analysis. Samples were taken in October and November of 2007 and early June of 2008 to provide some evidence of seasonal differences. *Thelypteris noveboracensis* grows by a spreading rhizome and each plant produces an extensive patch of growth. For example, at Black Rock Forest (Cornwall, NY), an expansive patch occupying ca. 0.1 km^2 (10 ha) developed in a forest clearing within several seasons after opening of the canopy (Schuster, pers. comm., 2008).

Soil analyses.—Moisture content (expressed as % w/w) was determined gravimetrically by difference in weight between the fresh sample and after drying to constant weight at 109°C. The pH of the soil samples in aqueous suspension (5 g per 50 ml distilled water) was obtained using an Accumet™ model 15 pH meter (Fisher Scientific, Fairlawn, NJ). Percent (w/w) organic content was determined by difference in weight between the fresh sample and after combustion at 375°C for 12–16 hours.

Microbiota.—Densities of bacteria that typically serve as prey for the eukaryotic microbiota were estimated by direct fluorescent microscopic counting (Anderson *et al.*, 2001). Microflagellate densities in aqueous extracts of the soil samples, fixed in 2% TEM grade glutaraldehyde, were determined using an acridine orange fluorescence method (Anderson *et al.*, 2001) adapted from Hobbie *et al.*, 1977. Testate amoebae and ciliates were enumerated by exhaustive examination of 3 ml (50 µl subsamples per observation) of aqueous-suspended soil samples fixed with 2% TEM grade glutaraldehyde and stained with Lugol's iodine (Anderson, 2008). Densities of naked amoebae were estimated using a standard culture observation method (COM), and cyst densities were estimated using the dried aliquot culture observation method

(DCOM) as published previously (Anderson, 2000). For the COM, a freshly collected sample of soil was suspended in micropore-filtered pond water (MFPW) in a ratio of 1 g in 50 to 80 ml total, and thoroughly dispersed. A 10 µl aliquot was dispensed per well of a 24-well Falcon™ tissue culture dish containing 2 ml of MFPW and a small cube of malt yeast agar (MYA) serving as a nutrient source for prey bacteria. Triplicate plates were prepared for each sample. After 10 to 14 days incubation at 25°C, each well was examined to determine the presence or absence of a given amoeba morphospecies indicating, if present, that at least one individual of that morphospecies was in the 10 µl aliquot. The total tally of each morphospecies was obtained and converted to number per ml of the original suspension. The number of amoebae per g dry weight of soil was calculated based on the weight of soil used to make the original suspension (Anderson, 2000). For the DCOM method, a 10 µl aliquot of the soil suspension was deposited in each of the dry wells of the Falcon culture dish and rapidly dried by flowing air before the MYA and 2 ml of MFPW were added. Thus, only the encysted amoebae survive the drying; and their count, based on observations of emergent morphospecies in the wells of the tissue culture dish, indicates the density of encysted amoebae in the original soil sample. Diversity of naked amoebae morphospecies was determined using the Shannon-Wiener formula ($H = 1 - \Sigma\, p_i \cdot \log_2 p_i$.) where p_i is the proportion of each morphospecies relative to the total. All microscopic observations were made with a Nikon Diaphot™ inverted compound microscope using phase contrast optics.

Results

Fern soil moisture at the sampling site ranged from 30 to 34%, pH was 4.4, and the organic content of the soil was 20%. Densities (number/g dry weight) of bacteria ranged from 2.5 to 7.1×10^9 comparable to published data for other terrestrial sites. The densities of protozoa in the autumn and June samples are presented in Table 1, including the percentages (in parentheses) of naked amoebae that were encysted and of testate amoebae that were atrophied or present as empty shells. No ciliates were observed in the Lugol's iodine preserved samples, which is consistent with other reports that a significant number of soil ciliates are usually encysted under typical field conditions, except when soil water is elevated following heavy precipitation (e.g., Foissner, 1987). However, as observed with terrestrial moss samples (Anderson, 2008), occasional ciliates were observed in the COM culture wells, confirming that encysted ciliates were probably present and became active when more fully hydrated. The diversity of naked amoebae was relatively high (H = ca. 3.0). The major genera of naked amoebae identified included *Acanthamoeba*, *Arachnula*, *Cochliopodium*, *Hartmannella*, *Mayorella*, *Saccamoeba*, *Thecamoeba*, *Vahlkampfia*, *Vexillifera* and *Vannella*, further indicating relatively rich species diversity. The major genera of testate amoebae were *Trinema*, *Euglypha*, *Tracheleuglypha*, *Corythion* and occasionally *cryptodif-*

TABLE 1. Densities of microbiota in fern soil samples during autumn 2007 and June 2008.

Sample date	Micro-flagellates	Naked amoebae	Testate amoebae
Oct. 24	6.5×10^6	4.0×10^6 (53%)	400 (66%)
Nov. 8	9.8×10^6	2.1×10^6 (48%)	444 (80%)
June 7	1.3×10^8	1.8×10^3 (50%)	400 (20%)

Densities are number/g dry weight of soil. All microflagellates counted appeared to be trophic stages; the percentages of encysted naked amoebae and of testate amoebae with empty shells or atrophied cytoplasm are presented in parentheses.

flugia. A globose testate amoeba, possibly *Geopyxella*, was occasionally abundant.

Interestingly in this study, the density of naked amoebae in the June sample (ca. 2×10^3) was less than in the October and November samples (ca. 4.0 and 2.0×10^6, respectively). However, the microflagellate density was substantially higher in June (ca. 1×10^8) compared to the October and November samples (6.5 and 9.8×10^6, respectively). While the total density of testate amoebae was relatively constant for the three sampling dates (ca. 400), the percent inactive was lowest in June (20%). Comparative data with other plant groups obtained from some representative published sources, including terrestrial mosses and the rhizosphere of seed plants, are presented in Table 2. In general, the densities of flagellates and naked amoebae were within the broad range found in other plant communities, but the testate amoeba densities were somewhat lower.

DISCUSSION

This research presents some of the first data on eukaryotic microbial organisms associated with the rhizosphere of a temperate fern in a northeastern U.S.A. hardwood forest. The robust growth of the fern's creeping rhizome, and production of organic matter due to secretory products, exfoliation of rhizome scales, deposition of shed fronds, etc., may contribute to the relative high organic content of the rhizosphere soil (20%) and resulting relatively high moisture content (ca. 30%), thus supporting a robust microbial community. Overall, the densities of fern-associated protozoa are reasonably similar to that found in other plant communities, but as discussed below in some cases they exceeded the densities associated with seed plants in organically enriched soils. The published data on seed plant rhizosphere microbiota encompasses a broad range of habitats including deserts, cultivated soils, grasslands, temperate forests, and tropical rain forests (Table 2). A more informed analysis is possible when the data from the current study are compared to some illustrative published results from organically rich soils. For example, in organically rich soils, the reported density of flagellates was in the range of 10^5, and naked amoebae 1.4 to 2.6×10^4 (Griffiths, 1990), or as much as 2×10^5 in tropical soil litter (Bamforth, 2006). Clarholm (1994) reported peak flagellate densities of ca. 4×10^6 in litter of a pine forest following rain

TABLE 2. Densities of microbiota in the fern rhizosphere compared to some published data for other plant communities.

Plant group	Terrestrial Mosses[1]	Fern *T. noveboracensis*	Seed plants[3]
Flagellates	1×10^5–4×10^7 (Anderson, 2008)	1×10^7–1×10^8	1.3×10^5–4×10^6 (Griffiths, 1990; Clarholm, 1994)
Naked amoebae	3.5×10^3–3.6×10^4 (Anderson, 2006, 2008)	2×10^3–4×10^6	2×10^3–2×10^6 (Clarholm, 1989, 1994; Griffiths, 1990; Bamforth, 2006)
Testate amoebae	3×10^2–6×10^3 (Anderson, 2008)	90–300[2]	1.3×10^4–3.7×10^5 (Lousier, 1982; Wanner and Xylander, 2005; Bamforth, 2006)

[1] Data are from published sources on terrestrial mosses in Torrey Cliff, NY and Toolik, AK.
[2] Adjusted data for living individuals, excluding those atrophied or with empty shells.
[3] Data for seed plants are largely from cultivated soils and tree-bearing sites.

during September 1977, but the lowest values were on the order of 2×10^6. Both the flagellates and naked amoebae in the fern soil examined in this study reached densities substantially higher, including published data for terrestrial mosses (Anderson, 2006, 2008). The densities and diversity of testate amoebae are less than those reported in other terrestrial sites, although the genera detected are not particularly unusual for natural soils (Foissner, 1987; Cowling, 1994).

Overall, this study indicates that *T. noveboracensis*, at least at the Torrey Cliff site, sustains a rich and substantial community of soil microbiota. Additional research is needed to replicate this work at other geographic locations, including other fern species, to more fully document fern rhizosphere microbiota. However, these data provide at least the first evidence of protozoan densities in a fern rhizosphere at a temperate woodland site. In general, there is limited biogeographic data on terrestrial microbial communities associated with diverse plants and much additional systematic research is needed to more fully understand the population structure and ecological dynamics of plant-associated microbial communities on a global scale.

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